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Occurrence of *Fusarium* spp. and Fumonisin in Durum Wheat Grains

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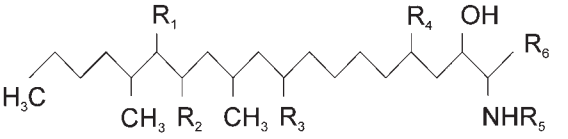
ABSTRACT: A survey was carried out to determine *Fusarium* species and fumonisin contamination in 55 durum wheat (*Triticum turgidum* L. var. *durum*) samples collected during two harvest seasons (2007 and 2008) using HPLC and further LC−MS/MS confirmation. All samples showed *Fusarium* contamination with infection levels ranging from 8 to 66%, *F. proliferatum* being the species most frequently isolated during 2007 and the second most frequently isolated one during the 2008 harvest season, respectively. Natural contamination with fumonisins was found in both harvest seasons. In 2007, 97% of the samples showed total fumonisin (FB_1 + FB_2) levels ranging from 10.5 to 1245.7 ng/g, while very low levels of fumonisins were detected in samples collected during 2008. These results could be explained by differences in the amount of rainfall during both periods evaluated. A selected number (n = 48) of *F. proliferatum* isolates showed fumonisin production capability on autoclaved rice. This is the first report of the presence of natural fumonisins in durum wheat grains.

KEYWORDS: *Fusarium*, fumonisins, durum wheat, *Fusarium proliferatum*

INTRODUCTION

Fumonisins are mycotoxins produced by a variety of fungi of the *Fusarium* genus. These toxins are polyketides which are structurally similar to sphinganine and disrupt sphingolipid metabolism; this disruption might lead to the fumonisin-induced mycotoxicoses (Figure 1). Dietary exposure to these mycotoxins can cause various adverse health effects in animals and humans, including equine leukoencephalomalacia (ELEM) and porcine pulmonary edema. With respect to humans, studies on the prevalence of esophageal cancer in regions of South Africa, China, Italy, and Iran revealed an association between this disease and the consumption of corn contaminated by *Fusarium* spp.[1,2] Finally, fumonisins can cause neural tube defects in experimental animals, and thus may also have a role in human cases. It has been hypothesized that a cluster of anencephaly and spina bifida cases in southern Texas may have been related to fumonisins in corn products.[3,4] The International Agency for Research on Cancer (IARC) evaluated in 1992 the toxins derived from *Fusarium verticillioides* as possible carcinogens to humans. More recently, based on the research results obtained so far, FB_1 has been evaluated as a possible carcinogen to humans (class 2B).[5]

A wide range of surveys indicate that maize is the most important source of fumonisin contamination in human foods and animal feeds. In Argentina, corn and corn products showed different levels of contamination according to the region from which the samples were collected.[6−8] Several studies worldwide have reported fumonisins in different crops and foods including rice,[9] sorghum,[10] mung beans,[11] asparagus,[12] black tea,[13] cereals and animal feedstuff.[14] There is also evidence of fumonisin contamination in wheat grains and wheat-based products.[15−17] Shephard et al.[18] reviewed many of the reports on fumonisins in wheat and concluded that careful evaluation of the analytical methods and possible sources of contamination, as well as confirmation by appropriate and validated methods, is required in order to prevent the reporting of false positive results. However, low fumonisin levels in wheat grain and wheat-based foods in Italy, Spain and Japan have been reported and documented by liquid chromatography−mass spectrometry with selected ion monitoring.[15,16,19]



	R_1	R_2	R_3	R_4	R_5	R_6
FB_1	TCA	TCA	OH	OH	H	CH_3
FB_2	TCA	TCA	H	OH	H	CH_3
FB_3	TCA	TCA	OH	H	H	CH_3

Figure 1. Basic structure of fumonisin and position of R side chains of fumonisin B_1, B_2 and B_3.

Durum wheat (*Triticum turgidum* L. var. *durum*) is an important small grain cereal, used for human consumption. In Argentina durum wheat is mainly used for the elaboration of pasta. The national production of pasta reached 369,600 ton in 2008, and the consumption per capita was estimated at 8.5 kg/year.[20] The total durum wheat production is devoted to the local market since the decrease in the planted area in the last decades does not allow satisfying the demand of international markets. Durum wheat cultivation is concentrated in the south of Buenos Aires province, which belongs to the Pampas region.

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Fusarium graminearum Schw, perfect stage *Gibberella zeae* (Schw.) Petch. is the main pathogen associated with *Fusarium* head blight (FHB) in common and durum wheat in Argentina.[21,22] Also natural occurrence of deoxynivalenol has been reported in both common and durum wheat.[21,22] Ramirez et al. (personal communication) carried out a mycological survey during a non-FHB epidemic year in common wheat and found that the predominant *Fusarium* species were *Fusarium proliferatum*, *Fusarium subglutinans* and *Fusarium verticillioides*. However there is a lack of information about the *Fusarium* species found and their mycotoxins in durum wheat during a non-FHB epidemic year.

The aims of this study were as follows: to determine the *Fusarium* species present on durum wheat during two non-FHB epidemic years; to determine the natural occurrence of fumonisins in durum wheat; and to evaluate the fumonisin production of the isolated *Fusarium* strains. Our working hypotheses were that *Fusarium* isolates belonging to the *Gibberella fujikuroi* species complex are the predominant species found on durum wheat during a non-FHB epidemic year and therefore this crop might be contaminated with fumonisins.

MATERIALS AND METHODS

Wheat Samples. A total of 55 freshly harvested durum wheat samples (500 g) were randomly collected during two consecutive harvest seasons (2007 and 2008) in different commercial fields located in the major durum wheat production area in Argentina, south of Buenos Aires province (La Dulce, latitude 38°24′25.48″ S, longitude 58°40′07.27″ W, and Bahia Blanca, latitude 38°18′31.50″ S, longitude 59°23′28.66″ W). Samples were collected from each field and pooled; from this pool a subsample of 500 g was taken. These subsamples were immediately analyzed for fungal contamination and then stored at 4 °C until mycotoxin analyses. All wheat samples were asymptomatic (no evident kernel damage).

The region where the sampling was done has a cool to temperate climate. Meteorological data such as rainfall were registered periodically.

***Fusarium* Isolation and Identification.** From each sample 100 wheat grains were plated (10 grains per Petri dish) onto a modified pentachloronitrobenzene medium (PNBC). The PNBC plates were incubated at 24 °C for 7 days under 12/12 h photoperiod cold white and black fluorescent lamps. *Fusarium* species developing from the grains were then identified according to previous guidelines.[23] Representative cultures of the species isolated were grown from single conidia for 10−14 days on Petri dishes of carnation leaf agar (CLA) and potato dextrose agar (PDA) slants, at 24 °C with a 12/12 h photoperiod under cold white and black fluorescent lamps. Forty-eight putative *F. proliferatum* strains were further identified by determining their *Gibberella fujikuroi* mating population (MP). Crosses were made in triplicate on carrot agar using an established protocol[24] with standard testers as female parents and the uncharacterized field isolates as male parents. Crosses were examined weekly and were considered positive when mature dark blue perithecia were observed with oozing ascospores. The tester strains used were M-6992 (*MATD-2*) and M-6993 (*MATD-1*), supplied by Dr. J. F. Leslie, Department of Plant Pathology, Kansas State University, Manhattan, KS.

Wheat Fumonisin Extraction. The fumonisin analysis performed was based mainly on the method of Shephard et al.[25] as has been described by Doko et al.[26] Subsamples of about 100 g were finely ground in a Buehler laboratory mill and thoroughly mixed. Aliquots of the ground subsamples (25 g) were shaken with 50 mL of methanol/water (3:1) for 30 min and filtered through Whatman No. 4 filter paper. While the flow rate was maintained below 2 mL/min, 10 mL of the filtered extract was applied to a Bond-Elut strong anion-exchange (SAX) cartridge (Varian, Harbor City, CA) fitted to a Supelco solid-phase extraction (SPE) manifold (Supelco, Bellefonte, PA), previously conditioned by the successive passage of methanol (5 mL) and methanol/water (3:1, 5 mL). The cartridge was then washed with methanol/water (3:1, 8 mL) followed by methanol (3 mL), and fumonisins were eluted with 0.5% acetic acid in methanol (14 mL). The eluate was evaporated to dryness at 40 °C, under a moderate stream of nitrogen, and stored dry at 4 °C until HPLC analysis.

Fumonisin Detection and Quantitation. The residue after cleanup was redissolved in 200 μL of acetonitrile/water (1:1). An aliquot (50 μL) of this solution was derivatized with 200 μL of an *o*-phthaldialdehyde (OPA) solution. The fumonisin OPA derivates (50 μL solution) were analyzed by using reversed-phase HPLC/fluorescence detection system. The HPLC system consisted of a Hewlett-Packard 1100 pump (Palo Alto, CA, USA) connected to a Hewlett-Packard 1046A programmable fluorescence detector and a data module Hewlett-Packard Kayak XA (HP ChemStation Rev. A.06.01). The column used was a 150 mm × 4.6 mm i.d., 5 μm, Luna-Phenomenex C_{18}(2) with a 4 mm × 3 mm i.d., Security Guard cartridge of the same material (Phenomenex, Torrance, CA, USA). Methanol:0.1 M sodium dihydrogen phosphate (75:25, v/v) solution adjusted to pH 3.35 with orthophosphoric acid was used as the mobile phase, at a flow rate of 1.5 mL/min. Fluorescence of the fumonisin OPA derivatives was recorded at excitation and emission wavelengths of 335 and 440 nm, respectively. Fumonisins were measured as peak heights and compared with reference standard solutions of fumonisins B_1 and B_2 (Sigma Chemical Co, St. Louis, MO, USA). A mixed acetonitrile:water (1:1, v/v) stock solution of FB_1 and FB_2 containing 50 μg/mL of each toxin was prepared. Four mixed working calibrant solutions (0.25, 0.5, 1.0, and 2.0 μL/mL) were prepared by diluting an aliquot of the stock solution with the appropriate volume of acetonitrile:water (1:1, v/v) . FB_3 concentrations were estimated on the basis of the FB_2/FB_3 signal ratio derived from a reference solution provided as part of a AOAC/IUPAC interlaboratory study (PROMEC, Tygerberg, South Africa). Appropriate dilutions of standards and/or sample extracts were made with acetonitrile/water (1:1).

A recovery experiment was performed in triplicate by spiking 25 g of ground fumonisin-free wheat samples with FB_1 and FB_2 toxins at levels of 10, 100, and 500 ng/g. Spiked samples were left overnight at room temperature to allow solvent evaporation prior to proceed with the extraction step. Mean recoveries for FB_1 and FB_2 were 98.98% and 94.9%, respectively. The detection limit of the analytical method was 10 ng/g for FB_1 and 15 ng/g for FB_2. The repeatability expressed as RSD were in the range 2.2−12.4% for FB_1 and 4.5 to 14.4 for FB_2, respectively. Detection limits were 3 and 5 ng/g for FB_1 and FB_2, respectively, based on the signal-to-noise ratio (3:1), and the limit of quantitation was established as 3 times the limit of detection (10 and 15 ng/g).

Apparatus and Experimental Conditions for LC−MS/MS Analysis. All LC−MS/MS analyses were performed using a Waters 2695 Alliance HPLC (Waters Corporation, Milford, MA, USA) equipped with a Waters Alliance 2685 pump, a Waters Alliance 2695 autosampler, a diode array detector Waters 2996 PDA interfaced to a Quattro Ultima Platinum tandem quadrupole mass spectrometer with electrospray ionization (ESI) source. The column used was a 2.1 mm × 150 mm i.d., 3.5 μm, XBridge C18 with a 2.1 mm × 10 mm i.d., guard column of the same material (Waters, Milford, MA). The interfaces were operated in a positive ion mode. Nebulizer and desolvation gases were nitrogen heated to 150 and 200 °C, respectively. The capillary voltage was 3.00 kV. The nitrogen flows were adjusted to 104 and 678 L/h for cone and desolvation gases, respectively. Multiple-reaction monitoring (MRM) was used for toxin determination. The precursor peak $[M + H]^+$ of FB_1 (m/z 722) and two products peaks (m/z 334 and m/z 352) were monitored to accomplish both quantitation and qualification criteria. Data acquisition and processing were performed using Mass Lynx V.4.1,

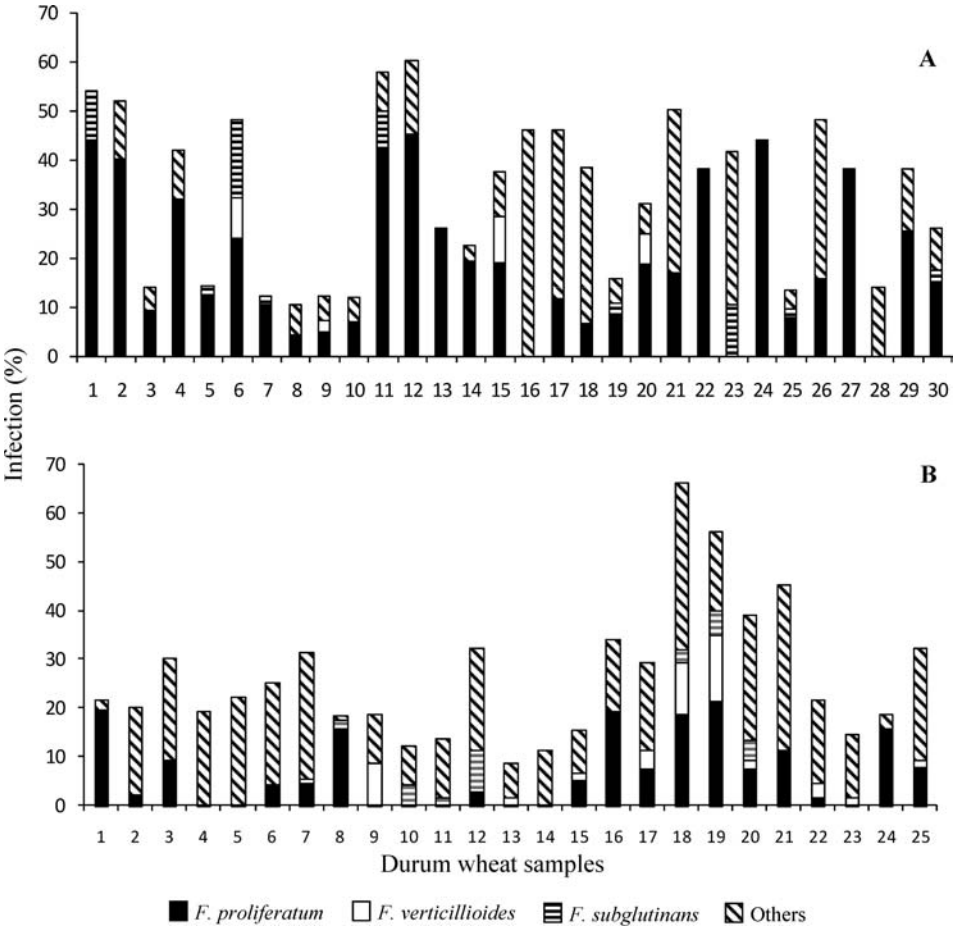

Figure 2. Infection percentage and distribution of *Fusarium* species in durum wheat samples. A: 2007 harvest season. B: 2008 harvest season. Others: mainly *F. equiseti, F. oxysporum, F. semitectum, F. graminearum, F. solani, F. poae*.

Waters INC software. Multiple reaction monitoring trace m/z 722 > 352 was used for quantitation.

The mobile phase of the chromatographic procedure was a gradient of aqueous 1% acetic acid/5 mM ammonium acetate (solvent A) methanol−1% acetic acid/5 mM ammonium acetate (solvent B). At the initial time, the eluant was 10.5% of solvent B. This composition was kept over 2 min, after which a linear gradient to 97.5% of solvent B was performed for 12 min, and then the eluant was kept at this composition for 3 min in order to clean the column. The initial conditions were stabilized over 5 min before the next injection. The flow rate was 0.2 mL/min. The column temperature was maintained at 22 °C. Aliquots of 30 μL of sample extracts were injected into the HPLC unit. Four points of identification were used to identify FB_1, i.e. retention time of 16.62 min, the precursor $[M + H]^+$ of m/z 722 and both product ions (m/z 334 and 352). A calibration curve was obtained injecting 10 μL of different FB_1 solution (0.25, 0.5, 1.0, and 2.0 μg/mL) in acetonitrile:water (1:1). Good linearity with a correlation coefficient higher than 0.996 was obtained for the calibration range. The calculated instrumental LOD (S/N = 3) for FB_1 was 0.01 ng/g and LOQ (S/N = 5) was 0.05 ng/g, and the relative within-day and between-day standard deviations (% RSD) were 6.5.

Fumonisin Production. Forty-eight *F. proliferatum* strains (*Gibberella fujikuroi* mating population D) isolated from the 2007 harvest season were selected for fumonisin production. Erlenmeyer flasks containing 100 g of rice and 40 mL of distilled water were autoclaved twice for 30 min at 121 °C. After cooling, rice was inoculated with an aqueous suspension of conidia (1 mL) of 10^6 spores obtained from CLA culture of each strain and incubated in the dark at 25 °C for 28 days. To avoid clump formation, the cultures were hand-shaken during the first days of incubation and thereafter as necessary. Rice cultures were then dried at 50 °C, finely ground with a laboratory mill, and stored at 4 °C until fumonisin analysis.

For each rice culture, a 15 g sample was extracted with 50 mL of acetonitrile:water (1:1) by shaking for 30 m and filtered through filter paper Whatman No. 4. An aliquot of the extracts (1000 μL) was taken and diluted with acetonitrile:water (1:1) as necessary for high performance liquid chromatography (HPLC) analysis. Fumonisins were determined as described above.

Statistical Analysis. To determine differences among fumonisin content in relation to the harvest seasons, the nonparametric test Kruscal−Wallis one way analysis of variance was used. Pearson correlation coefficient between fumonisin content and mean rainfall was calculated. All the studies were made using SigmaStat for Windows version 2.03 (SPSS Inc.). Statistical significance was determined at the level $P < 0.05$.

RESULTS AND DISCUSSION

Mycological analyses of the samples collected during 2 consecutive non-FHB epidemic years showed contamination with *Fusarium* species from all the durum wheat grain samples (Figure 2). During the first year (2007) the infection levels ranged from 10 to 60% while in the second year (2008) the infection levels ranged from 8 to 66%. The main *Fusarium* species

isolated was *F. proliferatum*, being the most frequently isolated species during 2007 and the second most frequently isolated one during the 2008 harvest season, respectively. Species isolated during the 2007 harvest season were *F. proliferatum*, *Fusarium equiseti*, *Fusarium oxysporum* and *Fusarium sublutinans*. Only three samples did not show contamination with *F. proliferatum*. However, during the 2008 harvest season the main *Fusarium* species isolated were *F. equiseti*, *F. proliferatum* and *F. oxysporum*. This is in agreement with Ramirez et al. (personal communication), who found that during a non-FHB epidemic year in common wheat the main *Fusarium* species identified belong to the *Gibberella fujikuroi* species complex. During FHB epidemic years the main *Fusarium* species isolated from common and durum wheat is *F. graminearum*.[21,22] The mains factors for FHB disease development are the environmental conditions, such as RH, rainfall and temperature, prevailing during the heading stage of the host plant. Severe FHB epidemics occurred in 1963, 1976, 1978, 1985[27] and 2001[28] in the main durum wheat growing area of Argentina. According to Kikot et al.,[29] using a weather-based predictive model, during the 2007 growing season the weather conditions were adverse for producing FHB infection events in the sampling area. This situation could explain the low percentage of *F. graminearum* isolates found from durum wheat grain samples during the present work.

In both harvest seasons studied, natural fumonisin contamination was present. However, fumonisin levels were higher in the first than in the second year. In the first harvest season (2007), 29 out of 30 samples (97%) were positive for fumonisins (B_1 and B_2) by HPLC analysis (Table 1). Total fumonisin level ranged from 10.5 to 1245.7 ng/g. Only one sample was higher than 1000 ng/g. FB_1 level ranged from 10.5 to 987.2 ng/g, and FB_2 level ranged from 15 to 258.5 ng/g. The FB_2/FB_1 ratio ranged from 0.1 to 2.3, with four samples containing more FB_2 than FB_1. Three samples showed contamination only with FB_1, and five samples showed similar occurrences. The presence of FB_1 was confirmed by LC−MS/MS analysis in all positive samples. In the second year (2008) none of the 25 samples analyzed showed fumonisin contamination by HPLC analysis, however further LC−MS/MS analysis demonstrated that only 6 samples showed traces (between LOD and LOQ) of fumonisin contamination . Comparison between the two harvest seasons showed significant differences ($p \leq 0.005$) in fumonisin content, and a positive correlation ($R = 0.5$, $p \leq 0.0001$) was also found between the mean rainfall (mm) and fumonisin levels. Although it was found that all the samples from both harvest seasons showed *Fusarium* spp. contamination at similar levels, the fact that only fumonisin contamination was found during the 2007 harvest season can be explained by differences in the amount of rainfall during both cultivation periods evaluated. The mean annual rainfall values registered between June and October (cultivation period) were 332 and 124 mm for the 2007 and 2008 harvest seasons, respectively. This difference was due to the fact that, during 2008, Buenos Aires province suffered the worst drought in the last seven decades.[30]

There are several reports in the literature about natural fumonisin contamination in bread and other varieties of wheat and in wheat-based products. In Spain, Castellá et al.[14] studied the natural occurrence of FB_1 and FB_2 in 17 wheat samples by HPLC analysis and confirmed these results by LC/MS. They found that 8 out of 17 wheat samples showed FB_1 contamination in a range of 200 to 8800 ng/g. In another survey on natural occurrence of FB_1 and FB_2 carried out in Italy by Cirillo et al.,[16] in 17 durum wheat pasta samples, FB_2 was only found in all samples analyzed in a range of 80 to 790 ng/g. The determination was done by LC−MS. Also in Italy, Castoria et al.[19] confirmed, by HPLC−MS, the presence of natural FB_1 and FB_2 contamination, in 5 out of 8 Farro samples (*Triticum monococcum* L., *Triticum dicoccon* Schrank and *Triticum spelta* L.) in low concentrations, up to 70 ng/g for FB_1, and FB_2 level was below the LOQ. Recently, Kushiro et al.[15] found natural FB_1 contamination in 1 out of 47 wheat samples collected in various areas of Japan, by LC−MS/MS, at trace level (between the LOD and the LOQ). The fumonisin levels in the present survey are comparable to the levels detected in the studies described above. Because nonchromatographic methods frequently produce false positive results,[18] and because the finding of fumonisins in wheat and wheat-based products is rather questionable, requiring a careful evaluation of the analytical methods used and a confirmation by appropriate methods,[31] during the present survey we used a HPLC method with further LC−MS/MS confirmation.

Due to the fact that *F. proliferatum* was the most frequent species isolated from the 2007 harvest season, we decided to study the ability of some selected strains ($n = 48$) isolated during

Table 1. Fumonisin Occurrence in 30 Durum Wheat Samples Collected in Buenos Aires Province (Argentina) from the 2007 Harvest Season

	concn (ng/g)			
sample	FB_1	FB_2	FB_2/FB_1	total fumonisins (ng/g)
1	300.0	82.0	0.273	382.0
2	79.0	31.0	0.39	110.0
3	58.0	27.0	0.46	85.0
4	39.6	35.8	0.90	75.4
6	56.3	33.1	0.58	89.4
7	288.1	179.9	0.62	468.0
8	10.5	nd[a]		10.5
9	75.7	20.6	0.27	96.3
10	399.6	84.8	0.21	484.4
11	36.1	30.8	0.85	66.9
12	16.2	19.1	1.17	35.3
13	12.0	28.0	2.33	40.0
14	18.3	nd		18.3
15	12.7	17.1	1.34	29.8
16	13.4	nd	nd	13.4
17	13.2	16.7	1.26	29.9
18	48.0	21.3	0.44	69.3
19	987.2	258.5	0.26	1245.7
20	nd	nd		nd
21	19.7	nd		19.7
22	241.9	82.9	0.34	324.8
23	721.8	204.6	0.28	926.4
24	70.4	25.1	0.35	95.5
25	786.9	137.8	0.17	924.7
26	307.3	47.9	0.15	355.2
27	18.8	19.7	1.04	38.5
28	599.8	141.8	0.23	741.6
29	14.0	15.9	1.13	29.9
30	372.0	92.4	0.24	464.4

[a] Not detected, <10 ng/g for FB_1 and <15 ng/g for FB_2.

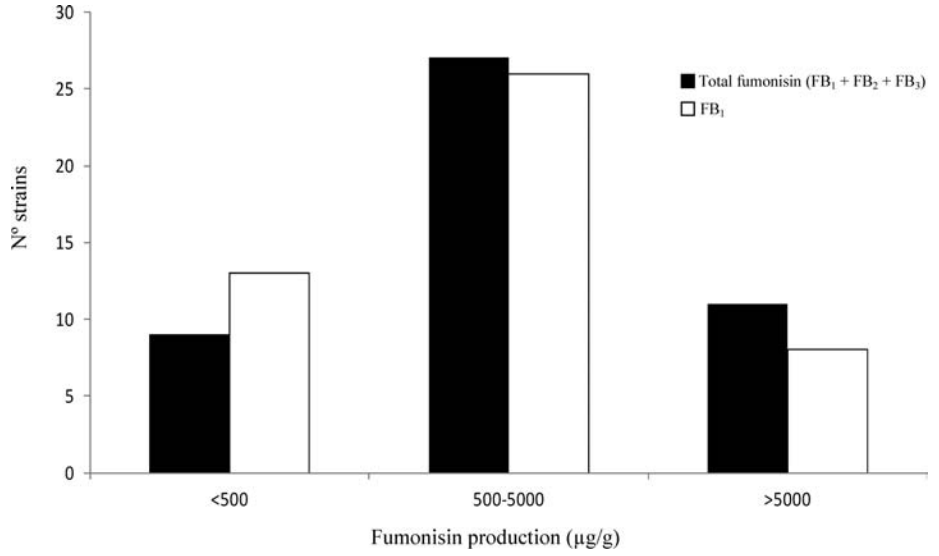

Figure 3. Total fumonisin (FB_1 + FB_2 + FB_3) and FB_1 production by 48 *Fusarium proliferatum* strains.

this harvest season, to produce FBs. All the putative *F. proliferatum* strains selected (n = 48) belonged to *Gibberella fujikuroi* mating population D and both mating types (*MATD-1* and *MATD-2*) were found among the toxigenic strains. The study revealed that 96% of the *F. proliferatum* strains isolated were able to produce FBs in variable levels on autoclaved rice. Total fumonisin production varied from 6.5 to 19326.7 μg/g, FB_1 being the most abundant (Figure 3). Forty-four out of 48 strains produced FB_1 in levels ranging from 6.5 to 11773.3 μg/g. Forty strains out of 48 produced FB_2 in a range from 4.4 to 5220.8 μg/g, and 33 strains out of 48 produced FB_3 in levels ranging from 5 to 2590.8 μg/g. The FB_2/FB_1 ratio ranged from 0.1 to 0.8, and the FB_3/FB_1 ratio ranged from 0.003 to 0.4. There were 5 strains out of 48 that produced only FB_1 and one out of 48 that produced only FB_2. The FB_3/FB_2 ratio ranged from 0.1 to 2.8, with 6 strains out of 48, producing more FB_3 than FB_2.

Desjardins et al.[32] showed that 9 strains of *F. proliferatum* (*Gibberella fujikuroi* mating population D) isolated from wheat kernels from Nepal produced fumonisins B_1, B_2 and B_3 in maize grains, in levels ranging from 48 to 1,060 μg/g. Also, Castellá et al.[33] studied the ability of 2 strains of *F. proliferatum* isolated from wheat to produce fumonisins in autoclaved corn kernels and found that both strains produced fumonisin in a range from 31.5 to 3.247 μg/g.

Although the ability of *F. proliferatum* strains to produce fumonisins in durum wheat was not evaluated in the present study, the fact that the toxin was found in naturally contaminated samples demonstrates that fumonisins can be synthesized in this cereal, and *F. proliferatum* could be one of the main species responsible for fumonisin contamination.

It is clear that fumonisin contamination is found in durum wheat during non-FHB epidemic years and when the conditions were appropriate. From the ecological point of view, it would be adequate to search on the optimal conditions for growth and fumonisin production in this substrate by *F. proliferatum*.

This is the first report of natural fumonisin occurrence in durum wheat grains in Argentina; based on this result we consider that it is necessary to conduct further studies in different areas where this cereal is cultivated, overall in years where the conditions are not inductive for FHB. It would also be necessary to evaluate the population risk of fumonisin exposure, mainly in those countries where pasta consumption is high.

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